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<b>(21) International Application Number:</b> PCT/AU95/00692 <b>(22) International Filing Date:</b> 19 October 1995 (19.10.95)  <b>(30) Priority Data:</b> PM 8906 19 October 1994 (19.10.94) AU  <b>(71) Applicant (for all designated States except US):</b> INSTITUTE FOR CHILD HEALTH RESEARCH [AU/AU]; Roberts Road, Subiaco, W.A. 6008 (AU).  <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only):</b> SANDERSON, Colin, John [AU/AU]; 10 Chiswick Street, Wembley Downs, W.A. 6019 (AU).  <b>(74) Agents:</b> HUGHES, E., John, L. et al.; Davies Collison Cave, 1 Little Collins Street, Melbourne, VIC 3000 (AU).	<b>(81) Designated States:</b> AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> CYTOASSAY  <b>(57) Abstract</b>  The present invention relates generally to a cytoassay. More particularly, the present invention relates to a system for assaying for cell viability and activity by detecting a signal from a non-indigenous reporter molecule in the cell. The cytoassay of the present invention is particularly useful in assays for cell growth, cytotoxicity and/or a cell adherence and for agonists or antagonists thereof.		

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## CYTOASSAY

The present invention relates generally to a cytoassay. More particularly, the present invention relates to a system for assaying for cell viability and activity by detecting a  
5 signal from a non-indigenous reporter molecule in the cell. The cytoassay of the present invention is particularly useful in assays for cell growth, cytotoxicity and/or a cell adherence and for agonists or antagonists thereof.

10 Bibliographic details of publications referred to herein are given at the end of the specification.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to  
15 imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Cell growth assays have been used to detect cytokines and their agonists and antagonists. A number of techniques are currently employed. One involves radioisotopes such as the  
20 incorporation of  $^3\text{H}$ -thymidine ( $^3\text{H}$ -Tdr) into DNA which relies on radioactivity measuring apparatuses, the most common being scintillation counters. Such counters have a wide dynamic range and the technique is accurate and sensitive in measuring cell growth. However, in addition to the general hazards of working with radioisotopes, the technique has a major disadvantage in that the cells need to be processed to separate  
25 unincorporated isotopic material prior to being prepared for liquid scintillation counting.

Another technique which is employed involves measurement of intracellular indigenous enzymes. This approach is predicated on finding substrates for intracellular enzymes which can be measured in a spectrophotometer (7). The dynamic range of the plate  
30 spectrophotometers is very low and errors are relatively high. Although the cells do not generally require further processing prior to spectrophotometry, extra steps are frequently required to solubilise the product before it can be determined on the plate reader.

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A further method involves determining cell growth by measurement of intracellular esterase with a fluorescent substrate and a plate fluorimeter (6). Although fluorimeters have a wide dynamic range, the substrates are unstable in aqueous solution and so the background readings can be high. Consequently, the sensitivity of this method is low  
5 and the cells require additional processing to remove exogenous esterases present in the medium.

A need exists, therefore, for an assay which is sensitive and which is not dependent on cell growth to determine cell viability or presence but which can be readily adapted as  
10 a cell growth assay.

In accordance with the present invention, the inventors have developed an assay based on the presence of a non-indigenous reporter molecule to detect cell activity. By "activity" as used herein is meant to include cell viability and is a measure of cell  
15 growth, survival and/or adherence to a solid support. The term "non-indigenous" in relation to a reporter molecule of the present invention means a molecule which is not naturally occurring within a cell and is thereby a non-native molecule. Generally, non-indigenous molecules are recombinant peptides, polypeptides or proteins and produce a detectable signal. Preferred reporter molecules are exogenous reporter enzymes capable  
20 of producing a detectable signal by metabolism of a substrate or are recombinant antigens useful in immune based assays.

Accordingly, one aspect of the present invention contemplates a method for detecting activity of an indicator cell line, said method comprising generating a transfectant of said  
25 cell line with a genetic sequence encoding a reporter molecule or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said reporter molecule genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit growth of said cells and then detecting cell growth by  
30 determining the presence of said reporter molecule.

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Generally, the reporter molecule:

- (i) is not indigenous to said cell;
- (ii) can be stably expressed by the cell;
- (iii) is capable of being detected by an assay system which provides a  
5 measure of cell viability of the said indicator line; and/or
- (iv) degrades following cell death.

This aspect of the present invention is particularly applicable for the development of a cell growth assay, especially of factor-dependent cell lines. Such an assay is applicable  
10 for screening for cytokines or agonists or antagonists thereof for said cell lines.

According to this embodiment there is provided a method for detecting growth of a factor-dependent indicator cell line, said method comprising generating a transfectant of said factor-dependent cell line with a genetic sequence encoding a reporter molecule or  
15 a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said reporter molecule genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line in the presence of a putative factor or sample containing a putative factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then  
20 detecting cell growth by determining the presence of said reporter molecule.

A "factor-dependent cell line" is a cell line which depends on the presence of a growth factor for survival and/or growth. Such cell lines include the B13 cell line (1) which grows in the presence of a number of mouse growth factors, including IL5. Other  
25 suitable cell lines are described by Greenberger *et al* (4) and Gillis *et al* (5). Further cell lines have been established by transfecting an existing cell line with a growth factor receptor so that the cell line becomes dependent on the ligand for the transfected receptor. Because the cell lines are dependent on a growth factor for their growth and/or survival they can be used to detect growth factors in biological fluids. Thus, cell lines  
30 are available or can be generated which will detect a large number of different growth factors.

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The indicator cell line could be derived from any animal species. This can be achieved by utilising a vector in which the reporter molecule gene expression is controlled by enhancers and a promoter which are functional in the required cell line. Useful promoter systems for mammalian cell lines include: the human cytomegalovirus (HCMV) enhancer and promoter (10), the promoter from the polypeptide chain elongation factor (EK-1 $\alpha$ ) gene (11), the promoter from the Simian Virus 40 (SV40) [12]. A particularly useful vector would also contain the sequences encoding a selectable marker and controlling sequences so that this gene is also expressed in the required cell line. A example of a selectable marker includes the *neo* gene which expression confers resistance to the drug G418 and allows selection of cells carrying the inserted vector.

After transfection and selection, a clone of the required cell line is chosen which shows the required growth characteristics of the parent cell line, and expresses the reporter molecule. The advantage of this assay is that after cell death the reporter molecules are preferably rapidly degraded thus the detection of reporter molecule activity provides an assay for the number of viable cells. This can be used in a quantitative assay whereby the activity per cell is determined by comparison of total reporter molecule activity in a determined number of cells. For example, the number of cells can be determined in a Coulter Counter. With the activity per cell determined the number of viable cells in a series of test samples can be determined by assay for reporter molecule activity.

For most applications quantitative determination is not required and assay results can be determined by comparison with positive and negative controls. A positive control would be indicator cells incubated under conditions to ensure maximum growth or viability, and a negative control would be indicator cells incubated under conditions required for minimal growth or viability.

The present invention is exemplified with reference to luciferase as the reporter molecule. This enzyme is a particularly suitable reporter protein for use in the methods of the present invention as it is capable of being detected by the production of photons from luciferin in the presence of ATP and CoA. In a preferred embodiment, the factor

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dependent cell lines express luciferase. The factor under test is diluted in a microplate, and washed indicator cells are added. After incubation to allow the cells to grow, typically for 24-48 hours, the wells are assayed for luciferase activity. Luciferase activity provides an estimate of the number of viable cells in each well. Reference  
5 hereinafter to luciferase as the reporter molecule is to be taken as exemplary of other suitable reporter molecules and all such reporter molecules are encompassed by the present invention. For example, the reporter protein  $\beta$ -galactosidase encoded by the *LacZ* gene of *E. coli* may also be used. This is a particularly useful reporter molecule as it may be detected by a number of fluorescent and chemiluminescent substrates.

10

Accordingly, another aspect of the present invention provides a method for detecting activity of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence  
15 is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit growth of said cells and then detecting cell growth by luciferase activity on luciferin.

Accordingly, this aspect of the present invention contemplates a method for detecting  
20 adherence of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed in said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient  
25 to permit adherence of said cells and then detecting luciferase activity on luciferin after non-adhered cells are removed.

Removal of cells may be by any convenient means such as but not limited to washing, dilution, centrifugation or by decanting. The present invention extends to a modified  
30 assay to screen for agonists and/or antagonist of cell adherence as well as to such agonists and antagonists when identified by the assay herein described.

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The present invention more particularly contemplates a method for detecting growth of a factor-dependent indicator cell line, said method comprising generating a transfectant of said factor-dependent cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that  
5 said luciferase genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line in the presence of a putative factor or sample containing a putative factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell growth by luciferase activity on luciferin.

10

As stated above, the luciferase gene catalyses the production of photons from luciferin in the presence of ATP and Coenzyme A. Conveniently, the genetic sequence is a cDNA encoding firefly luciferase, such as described by DeWet *et al.* (9) or a functionally active mutant, derivative, part, fragment, homologue or analogue thereof.  
15 Henceforth, all such mutants and the like of luciferase are encompassed by the term "luciferase". While firefly luciferase is particularly described herein, any number of other luciferase genes may be employed. Examples of alternative luciferase genes include the genes from *Vargula hilgendorfii* (2) or from *Renilla reniformis* (3). The cDNA is carried and expressed on a suitable expression vector. Alternatively, the cDNA  
20 is inserted downstream of an existing genomic promoter such that growth of said cells stimulates expression of the luciferase gene.

The luciferase genetic construct is transfected into the cell line by any number of suitable techniques such as by electroporation with stable transfectants being selected on  
25 a suitable medium.

The method of the present invention is particularly applicable in screening biological or chemical samples for potential factors required for growth of factor-dependent cell lines. Biological and chemical samples include culture supernatant, conditioned medium, tissue  
30 and cell extracts, tissue fluid, whole blood, serum, lymph, respiratory and other mucus fluid and secretory fluid as well as preparations of chemical compounds whether in substantially pure or impure form. The factor dependent cell lines are generally animal



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cell lines having a requirement for a known or unknown factor, generally a cytokine, to promote, initiate or enhance maintenance of growth. The animal cell lines may originate from humans, laboratory test animals (e.g. mice, rats, rabbits, guinea pigs), livestock animals (e.g. cows, sheep, goats, horses), birds, companion animals (e.g. dogs, cats) or  
5 captive wild animals. The cell lines may also be tumorigenic. Examples of factors on which the cell lines may be dependent include growth factors, colony stimulating factors and interleukins (e.g. IL-1 to IL-16).

Another aspect of the present invention is directed to a kit useful for detecting factors  
10 such as cytokines and/or agonists or antagonists thereto, said kit comprising in multi-component form, a first component comprising a factor-dependent cell line capable of producing luciferase in response to said factor and a second or further components comprising reagents for detecting activity of luciferase.

15 Yet another aspect of the present invention is directed to a cytokine or an agonist or antagonist thereof identified by the cell growth assay of the present invention.

Another form of the subject cytoassay is in cytotoxicity testing of indicator cell lines. In this aspect of the invention, the cell line need not be a factor dependent cell line.  
20 Cytotoxicity assays are frequently used to determine cell mediated cytotoxicity (i.e. the killing of a target cell by another cell such as a cytotoxic T cell) or to monitor the cytotoxic effects of compounds or antibodies. In this application, the target cell expresses the luciferase gene and the cytotoxic effect is assessed by assay for luciferase. Because luciferase is rapidly degraded following the death of a cell, only viable cells  
25 would give a luciferase signal, thus the proportion of cells killed in the cytotoxicity assay could be determined by comparison with controls.

Accordingly, another aspect of the present invention contemplates a cytotoxicity assay for an indicator cell line, said method comprising generating a transfectant of said cell  
30 line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating

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said transfected indicator cell line in the presence of a putative cytotoxic factor or sample containing a putative cytotoxic factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell growth by luciferase activity on luciferin. Putative cytotoxic factors include but are not limited to chemical compounds, antibodies, recombinant molecules, naturally occurring and optionally isolated molecules (e.g. from plants, coral or microorganisms) and pharmaceutical compounds. The method may also be modified to identify agonists or antagonists of known cytotoxic agents by screening for enhanced or diminished cytotoxicity in the presence of the known agent.

10

The present invention extends, therefore, to a cytotoxic agent or an agonist or antagonist thereof identified by the cytotoxic assay herein described.

Still a further embodiment of the cytoassay is a cell adherence assay. Cell adherence assays are used to determine the adhesion of an indicator cell to other cells or to surfaces. This system allows the determination of the molecules involved in the adherence phenomenon to be studied and identified. In this application, the indicator cells express the luciferase. The indicator cells are allowed to settle on to test surface (monolayer or cells or molecules coupled to the bottom of a tissue culture well. After washing the adhering cells are determined by assay for luciferase. These assays could be adapted to test for antagonists and agonists of adherence, by incorporating test compounds into the medium during the adherence assay. As the test could be confounded by direct toxicity of the compound towards the indicator cells, a toxicity control may need to be carried out where the indicator cells are incubated with the test compounds. Any loss of luciferase activity indicates toxicity.

In a particularly preferred embodiment, the luciferase genetic sequence is inserted in a gene encoding a known receptor inducible gene. The insertion is such that upon a ligand interacting with the receptor, the luciferase genetic sequence is expressed. In accordance with this embodiment, the receptor is for the factor upon which the cell line is dependent. Such engineered factor-dependent cell lines are particularly useful in screening for ligand or ligands for the receptor or screening for agonists or antagonists

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of ligand interaction with the receptor.

Yet another aspect of the present invention is directed to a factor-dependent cell line transfected with a genetic construct encoding a luciferase such that upon growth of said  
5 factor-dependent cell line, there is detectable luciferase activity. Preferably, the luciferase genetic construct is expressed upon activation of the factor-dependent cell line with the said factor. Even more preferably, the luciferase genetic sequence is fused to the genetic sequence encoding a receptor for said factor such that luciferase is produced upon interaction between the factor and its receptor. Most preferably, the factor is IL5  
10 and its receptor is the IL5 receptor (IL5R).

In a particular aspect of the present invention, the luciferase genetic sequence is inserted into a specific location such that it becomes a marker for alternative splicing. Most genes in higher eukaryotes contain intervening sequences (introns) between coding  
15 sequences (exons). The introns are removed from pre-mRNA by splicing so that the coding sequences become contiguous in mature RNA. Some pre-mRNA undergoes alternative splicing so that the different gene products are derived from a single gene. In accordance with the present invention, a cytokine receptor is selected which directs synthesis of a pre-mRNA prior to alternative splicing. One such cytokine receptor is the  
20 IL5 receptor  $\alpha$ -chain gene. The luciferase genetic sequence is inserted downstream of an exon so as to direct synthesis of a fusion protein. Such a construct provides a convenient assay for analogues or agonists of the splicing factors for that receptor as well as for a range of antagonists of cytokine receptor production.

25 Still, in a further aspect of the present invention, there is provided a genetic construct comprising first and second nucleotide sequences wherein the first sequence encodes a receptor for a factor and said second nucleotide sequence encodes luciferase or functional like molecule wherein upon expression of the first nucleotide sequence, said second nucleotide sequence is expressed to produce luciferase. Preferably, the second  
30 nucleotide sequence is placed at the 3' end of an exon on said first nucleotide sequence. The genetic construct of the present invention is particularly useful in the generation of factor-dependent indicator cell lines.

- 10 -

The present invention is hereinafter described with reference to a cell growth assay using a factor-dependent cell line. This is done, however, with the understanding that the present invention extends to the use of both factor-dependent and factor-independent cell lines and other assays such as a cytotoxic assay or a cell adherence assay.

5

The present invention is further described by the following non-limiting Figures and/or Examples. Reporter molecules other than luciferase may be employed using similar or analogous methods to those hereindescribed. The present invention extends to all suitable reporter molecules in addition to luciferase.

10

In the Figures:

**Figure 1** is a graphical representation showing luciferase activity in Baf cells.

15 **Figure 2** is a graphical representation of a comparison of luciferase activity with  $^3\text{H}$ -Tdr incorporation. - o - Luciferase; -  $\nabla$  -  $^3\text{H}$ -Tdr.

**Figure 3** is a graphical representation showing IL5 production from stimulated EL4.23 cells. -□- control; -■- PMA; -▲- cAMP; and -◆- PMA/cAMP.

20

**Figure 4** is a graphical representation showing titration of murine IL5 from Baculovirus system over three different experiments (-■-, -▲- and -●-).

25 **Figure 5** is a graphical representation showing titration of human IL5 from Baculovirus system over three different experiments (-■-, -▲- and -x-).

**Figure 6** is a graphical representation showing a comparison of luciferase systems. -■- Lab 201; and -▲- Promega.

30 **Figure 7** is a graphical representation depicting B13-Luc survival.

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**Figure 8** is a diagrammatic representation of plasmid pHCMV-Luc. PE, promoter early; PL, promoter late; P, promoter; Enh, enhancer.

5

## EXAMPLE 1

### CELL LINES

Studies were conducted in Baf-IL5 $\alpha$ R cells (8) as well as the B13 cell line (1). The latter cell line grows in the presence of a number of mouse growth factors, including IL5.

10

## EXAMPLE 2

### LUCIFERASE GENE

A luciferase gene in the form of cDNA (2) was ligated into the pHCMV expression vector. This vector was constructed so that the selectable marker *neo* would express in mammalian cells under the control of the SV40 promoter and the luciferase gene under the control of the cytomegalovirus promoter. Similar vectors for generating stable transfectants in eukaryotic cells are commercially available such as vectors pBK-CMV and pBK-RSV from Stratagene. A diagrammatic representation of pHCMV-Luc is shown in Figure 8.

20

### EXAMPLE 3

#### PRODUCTION OF CELLS PRODUCING LUCIFERASE

The luciferase expression vector of Example 2 was transfected into the factor-dependent  
5 cell line B13 of Example 1 by electroporation. Stable transfectants were selected in  
G418 under limiting dilution conditions. A sample of clones was tested for luciferase  
expression and those expressing high levels of IL5 (see Example 4) were selected for  
further analysis. One of these selected clones is designated (B13-Luc).

### EXAMPLE 4

#### ASSAY FOR IL5

A standard solution of human IL5 was titrated in a microplate and CTLL-IL5R-Luc cells  
(Example 3) added at  $10^3$ /well to give a total volume of 100  $\mu$ l. After 48 hours, the  
luciferase assay reagent (Promega; containing luciferin, Coenzyme A, ATP and buffer)  
15 with the addition of 0.5% v/v Triton X 100 to lyse the cells was added in a volume of  
25  $\mu$ l. The plate was immediately placed in a Packard Top Count to determine light  
emissions. The use of Coenzyme A changes the kinetics of the reaction to provide a  
stable light emission. Thus, injection apparatus normally associated with luminometers  
is unnecessary. An experiment comparing luciferase activity with  $^3\text{H}$ -Tdr incorporation  
20 is shown in Figure 2. This illustrates that luciferase activity using  $5 \times 10^3$  cells/well  
gives plateau levels of about 30,000 light units. These data indicate the very low  
background levels in the absence of IL5 and sensitivity at least as good as  $^3\text{H}$ -Tdr.

### EXAMPLE 5

#### DETECTION OF MOUSE AND HUMAN IL5 USING AN IL5 DEPENDENT CELL LINE EXPRESSING LUCIFERASE

The Baf-Luc cell line was used in this assay. These cells were obtained by stably  
transfecting an IL3-dependent mouse cell line with the human IL5 receptor  $\alpha$ -chain (cell  
line Baf-IL5 $\alpha$ R). The human  $\alpha$ -receptor chain confers to this cell line growth-  
30 responsiveness to both mouse and human IL5. The Baf-IL5 $\alpha$ R cells were then  
transfected with the plasmid pHCMV-Luc (Figure 8). Cells highly expressing luciferase  
were selected and cloned to produce the Baf-Luc cell line. These cells are dependent

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on IL5 for growth and proliferation and will express luciferase only when proliferating.

#### **Production of IL5 by EL4-23 cells**

EL4-23, a mouse thymoma cell line with high inducible IL5 expression, was stimulated  
5 with phorbol ester (PMA), cyclic-AMP or a combination of the two for 24 hours.  
Production of IL5 in the supernatant of the cell culture was assayed using the Baf-Luc  
system. Baf-Luc cells (5000 cells/well) were incubated in the presence of a serial  
dilution (1:2) of EL4-23 supernatants for 48 hours. Baf-Luc cell survival was  
determined by monitoring luciferase activity. The results are shown in Figure 3.

10

#### **Titration of IL5 expressed in a baculovirus system**

Mouse and human recombinant IL5 are routinely prepared using a baculovirus system.  
To titrate the relative units of IL5 in each preparation, serial dilution (1:2) of different  
batches of mouse or human IL5 were prepared and added to Baf-Luc cells. Cell  
15 survival was determined by monitoring luciferase activity. The relative amount of IL5  
in each preparation was determined by comparing the 50% end point of each titration  
curve. The results are shown in Figures 4 (mouse) and 5 (human).

The Baf-Luc system is very sensitive. Luciferase activity can be measured with as few  
20 as 100-250 cells/well. Two luciferase buffers were tested. The "Lab201 home-made"  
buffer was made up using firefly luciferase (Molecular Probes). The promega buffer  
was a ready-to-use solution provided by the supplier. Both buffers gave a similar  
response curve when used with a known number of IL5 stimulated Baf-Luc cells. The  
results are shown in Figure 6.

25

### EXAMPLE 6

#### A METHOD FOR DETECTING AND QUANTIFYING HAEMOPOIETIC CELL SURVIVAL IN RESPONSE TO IL5 IN A CO-CULTURE WITH A STROMAL LAYER USING CELLS EXPRESSING LUCIFERASE

The pro-B lymphocyte cell line, B13, was used in this assay. These cells were originally derived from Balb/C mouse bone marrow and are responsive to IL3, IL4 and IL5. The B13 cells were transfected by electroporation with the plasmid pHCMV-Luc. This plasmid consists of the firefly luciferase gene ligated into pHCMV which also contains  
neomycin. Cells highly expressing luciferase were selected with geneticin (G418) 0.8  
mg/ml and cloned to produce a stable cell line, B13-luc. A preparation of baculovirus  
expressed recombinant IL5 was incubated with a stromal layer comprising a  
subconfluent monolayer of Swiss 3T3 cells, a murine embryonic mesenchymal cell line.  
The stromal layers were extensively washed to remove excess IL5. B13-luc cells were  
added to IL5 treated and untreated stromal layers and the survival of these growth factor  
dependent cells was assessed after 24 hour in co-culture. B13-luc cell survival was  
determined by monitoring the luciferase activity of the co-culture in the presence of  
ATP-luciferin-CoA substrate. Luciferase activity is expressed as counts per second  
(LCPS). Results are shown in Figure 7.

20

### EXAMPLE 7

#### MODIFICATION TO LUCIFERASE ASSAY

A modification to the luciferase assay includes in the assay system all the components required to assay luciferase activity as well as an agent to release the luciferase enzyme  
from the cell. An example is to include 0.5% v/v Triton x 100. Thus, at the end of an  
incubation period when it is required to determine the number, or relative number, of  
viable cells in the samples, an aliquot of the assay reagent is added to each sample in  
the container that was used for the incubation. The luciferase activity can then be  
determined in a luminometer without the need to harvest or process the cells to extract  
the luciferase enzyme. The preferred method is to use microplates for the incubation  
and then to determine the light output in a luminometer designed to read directly the  
activity in these microplates. However, other centrifugations can be used or samples can

30



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be transferred from an assay container to a container suitable for measurement in a luminometer.

### EXAMPLE 8

#### 5 DETERMINATION OF VIABLE AND RELATIVE CELL NUMBERS

The present invention provides an assay to determine viable and relative cell numbers which have various applications. These applications include:

- 10 (a) Assays where it is required to determine cell growth or viability where only a single cell type is involved. Examples include the determination of cytokine activity using factor dependent cell lines and the determination of cytotoxic agents.
- 15 (b) Assays where it is required to determine cell growth or viability of one cell type in the presence of another cell type. Examples include adhesion of one cell type to another, and the determination of cell mediated cytotoxic activity on an indicator cell line.

### EXAMPLE 9

#### 20 IDENTIFICATION OF LUCIFERASE ACTIVITY IN Baf CELLS

Baf-luc cells were counted in a coulter counter and different number distributed into the wells of a microplate. Luciferase reagent was added and the light output determined in a luminometer. Fewer than 100 cells can be detected as significantly above background. The average light output/cell is 0.48 LU and this value can be used to quantify the  
25 number of cells. The results are shown in Figure 1.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The  
30 invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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12. Kaufman (1990) *Meth. Enzymol* **185**: 487-511.

## CLAIMS:

1. A method for determining the activity of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding a reporter molecule or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said reporter molecule genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit growth of said cells and then detecting cell activity by determining the presence of said reporter molecule.
2. A method according to claim 1 wherein the reporter molecule is an enzyme.
3. A method according to claim 2 wherein the reporter molecule is luciferase.
4. A method according to claim 1 or 2 or 3 wherein the indicator cell line is a factor-dependent cell line.
5. A method for detecting activity of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit growth of said cells and then detecting cell activity by the action of luciferase on luciferin.
6. A method for detecting growth of a factor-dependent indicator cell line, said method comprising generating a transfectant of said factor-dependent cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said

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transfected indicator cell line in the presence of a putative factor or sample containing a putative factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell growth by luciferase activity on luciferin.

7. A method according to any one of the preceding claims wherein the indicator cell line is the B13 cell line or Baf-II5d receptor cell line.

8. A method according to claim 5 or 6 wherein the luciferase genetic sequence is derived from firefly.

9. A method according to claim 5 or 6 wherein the luciferase genetic sequence is derived from *Vargula hilgendorfii*.

10. A method for detecting activity of a factor-dependent indicator cell line, said method comprising generating a transfectant of said factor-dependent cell line with a genetic sequence encoding a reporter molecule or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said reporter molecule genetic sequence is capable of being expressed in the presence of said factor and then incubating said transfected indicator cell line in the presence of a putative factor or sample containing a putative factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell activity by determining the presence of said reporter molecule.

11. A method for detecting activity of a factor-dependent indicator cell line, said method comprising generating a transfectant of said factor-dependent cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed in the presence of said factor and then incubating said transfected indicator cell line in the presence of a putative factor or sample containing a putative factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell activity by the action of luciferase on luciferin.

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12. A method according to claim 11 wherein the luciferase genetic sequence is inserted into a genomic location such that it is a marker for alternative splicing.

13. A method according to claim 12 wherein the luciferase genetic sequence is inserted downstream of an exon in a gene encoding a cytokine receptor.

14. A method according to claim 12 wherein the luciferase genetic sequence is inserted downstream of an exon in a gene encoding the IL5 receptor  $\alpha$ -chain.

15. A factor-dependent cell line transfected with a genetic construct encoding a luciferase such that upon growth of said factor-dependent cell line, there is an increase in luciferase activity.

16. A factor-dependent cell line according to claim 15 wherein the luciferase genetic construct is expressed upon activation of the factor-dependent cell line with said factor.

17. A factor-dependent cell line according to claim 16 wherein the luciferase genetic sequence is fused to the genetic sequence encoding a receptor for said factor such that luciferase is produced upon interaction between the factor and its receptor.

18. A factor-dependent cell line according to claim 17 wherein the factor is IL-5 and its receptor is the IL-5 receptor.

19. A genetic construct comprising first and second nucleotide sequences wherein the first sequence encodes a receptor for a factor and said second nucleotide sequence encodes luciferase or functional like molecule wherein upon expression of the first nucleotide sequence, said second nucleotide sequence is expressed to produce luciferase.

20. A genetic construct according to claim 19 wherein the second nucleotide sequence is placed at the 3' end of an exon on said first nucleotide sequence.

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21. A kit useful for detecting factors such as cytokines and/or agonists or antagonists thereto, said kit comprising in multi-component form, a first component comprising a factor-dependent cell line capable of producing luciferase in response to said factor and a second or further components comprising reagents for detecting activity of luciferase.

22. A cytotoxicity assay for an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding a reporter molecule or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said reporter molecule genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line in the presence of a putative cytotoxic factor or sample containing a putative cytotoxic factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell activity by determining the presence of said reporter molecule.

23. A cytotoxicity assay for an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed upon growth of said indicator cell line and then incubating said transfected indicator cell line in the presence of a putative cytotoxic factor or sample containing a putative cytotoxic factor for a time and under conditions sufficient to permit growth of said cells if said factor is present and then detecting cell activity by the action of luciferase on luciferin.

24. A cytotoxicity assay according to claim 22 or 23 wherein putative cytotoxic factors are chemical compounds, antibodies, recombinant molecules, naturally occurring or synthetic isolated molecules and pharmaceutical compounds.

25. A method for detecting adherence of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding a reporter molecule or a functional mutant, derivative, part, fragment, homologue or

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analogue thereof such that said luciferase genetic sequence is capable of being expressed in said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit adherence of said cells and then detecting the presence of said reporter molecule after non-adhered cells are removed.

26. A method for detecting adherence of an indicator cell line, said method comprising generating a transfectant of said cell line with a genetic sequence encoding luciferase or a functional mutant, derivative, part, fragment, homologue or analogue thereof such that said luciferase genetic sequence is capable of being expressed in said indicator cell line and then incubating said transfected indicator cell line for a time and under conditions sufficient to permit adherence of said cells and then detecting luciferase activity on luciferin after non-adhered cells are removed.

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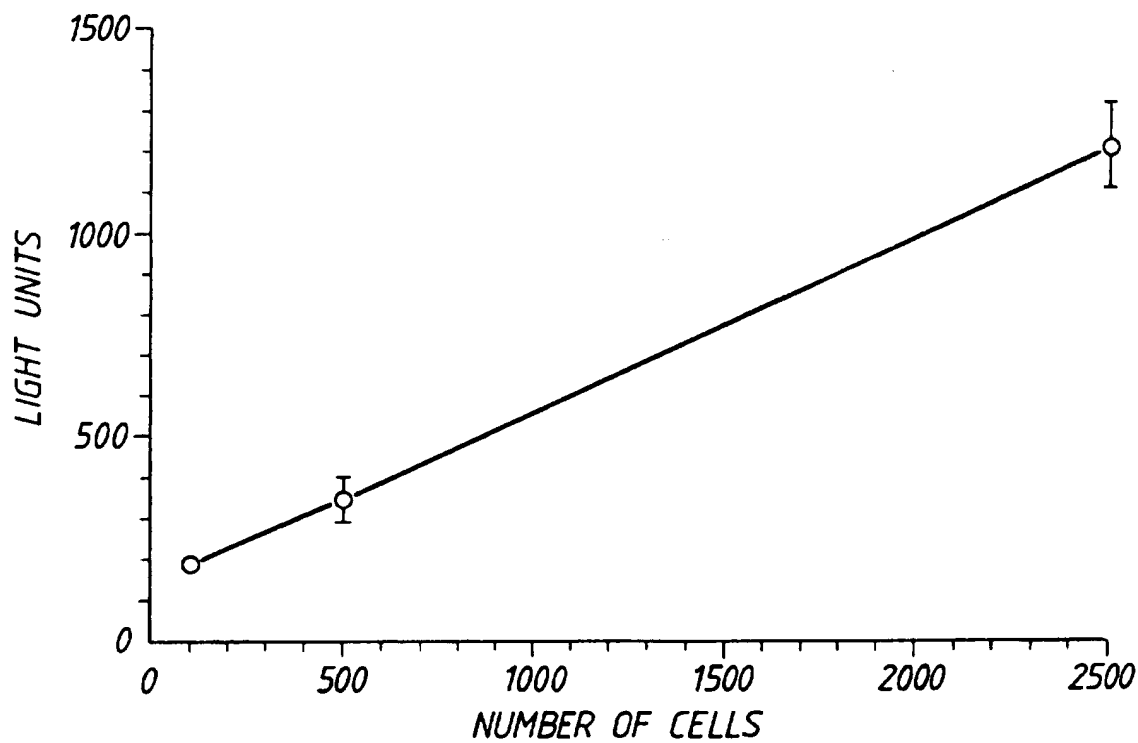


Fig.1

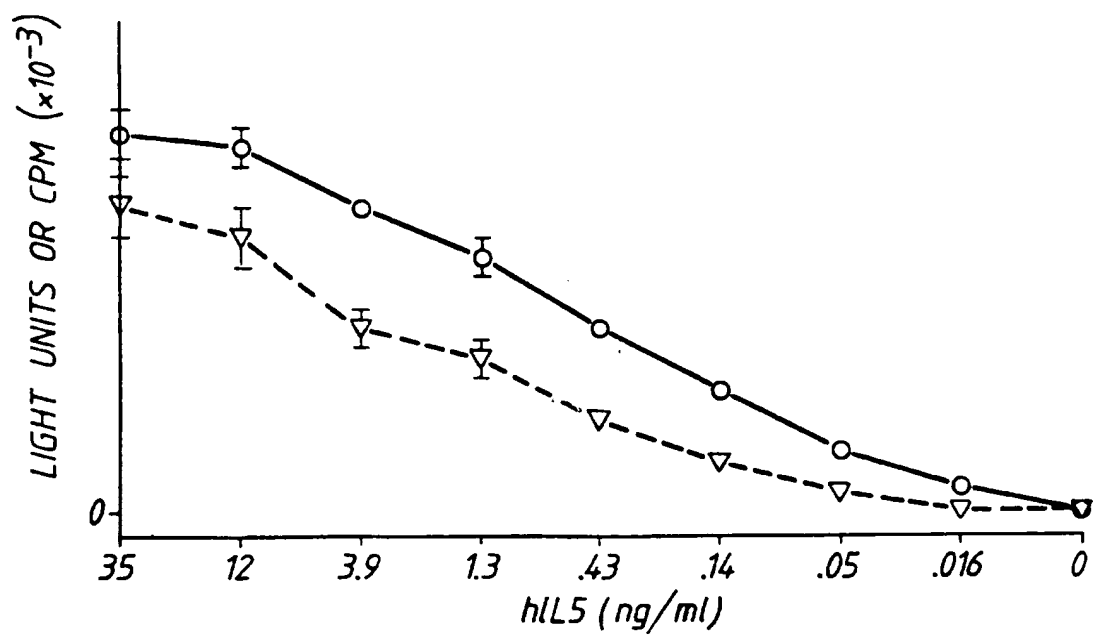


Fig.2



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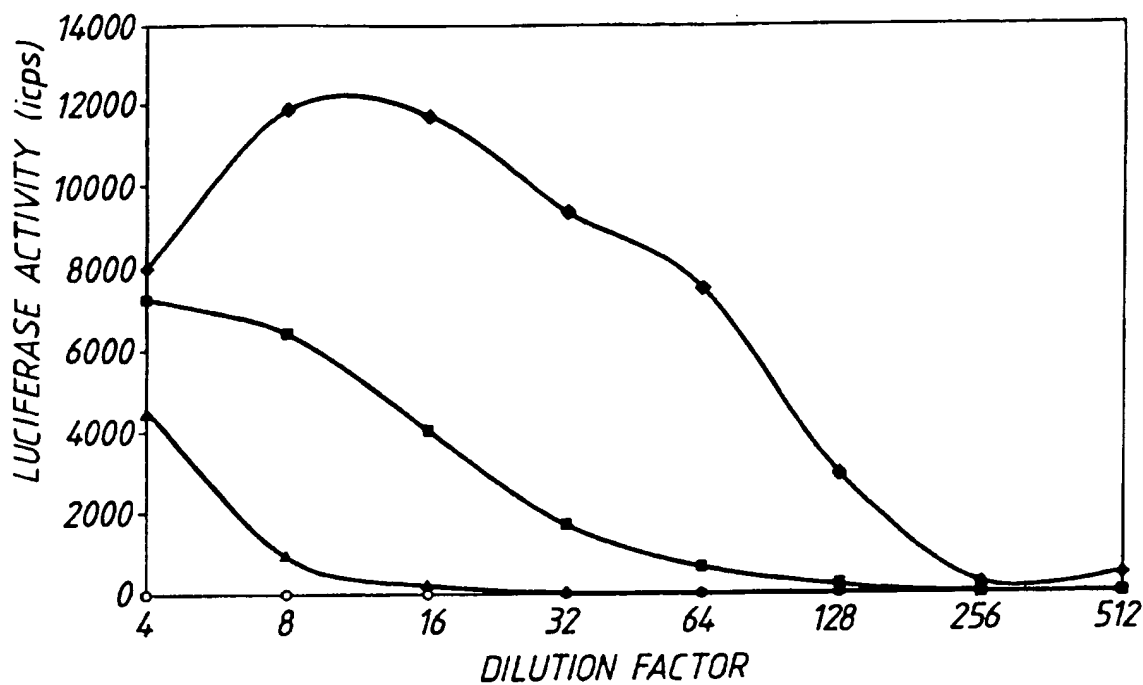


Fig.3

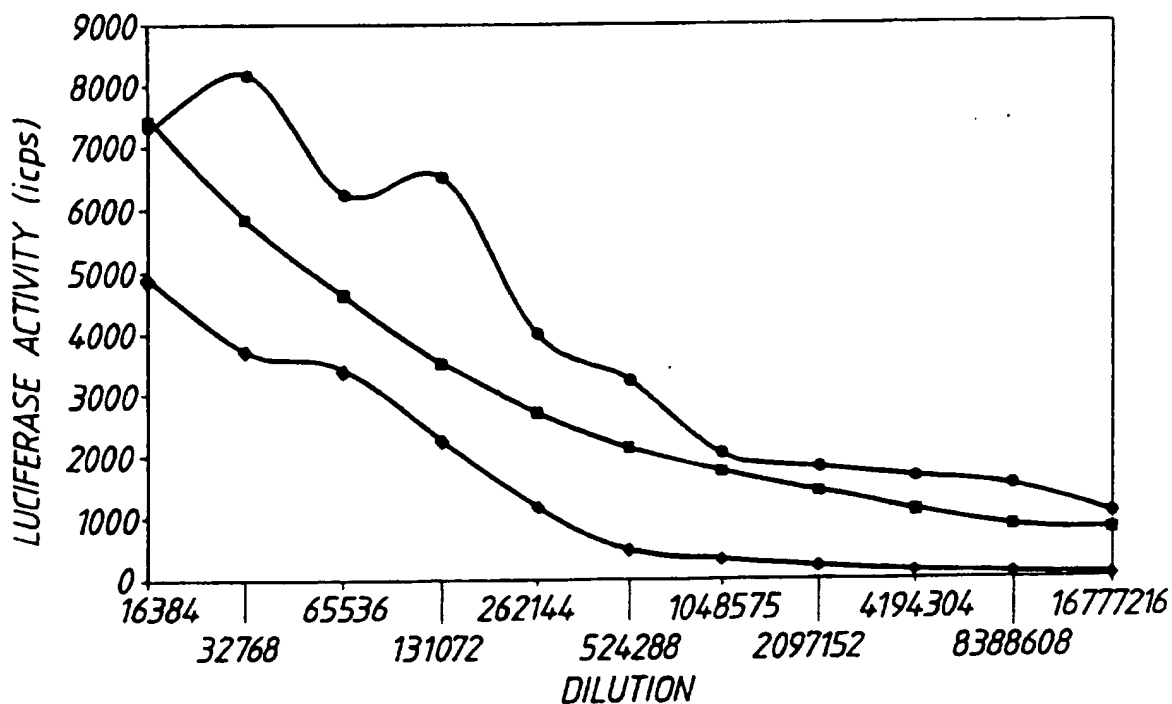


Fig.4

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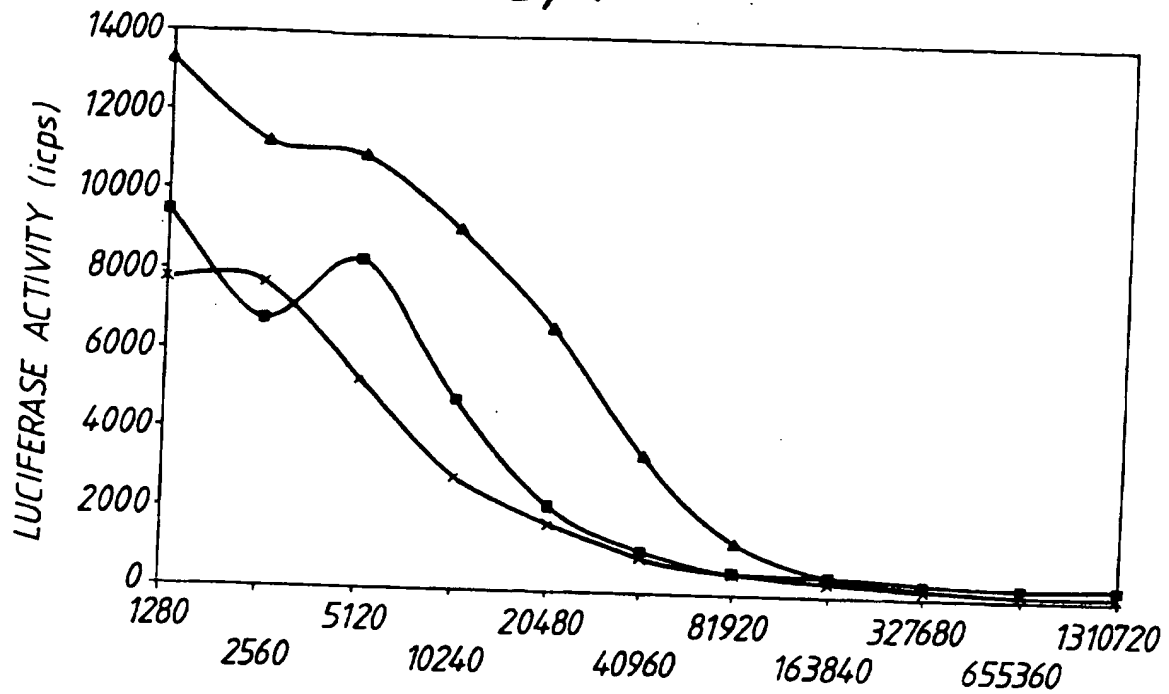


Fig.5

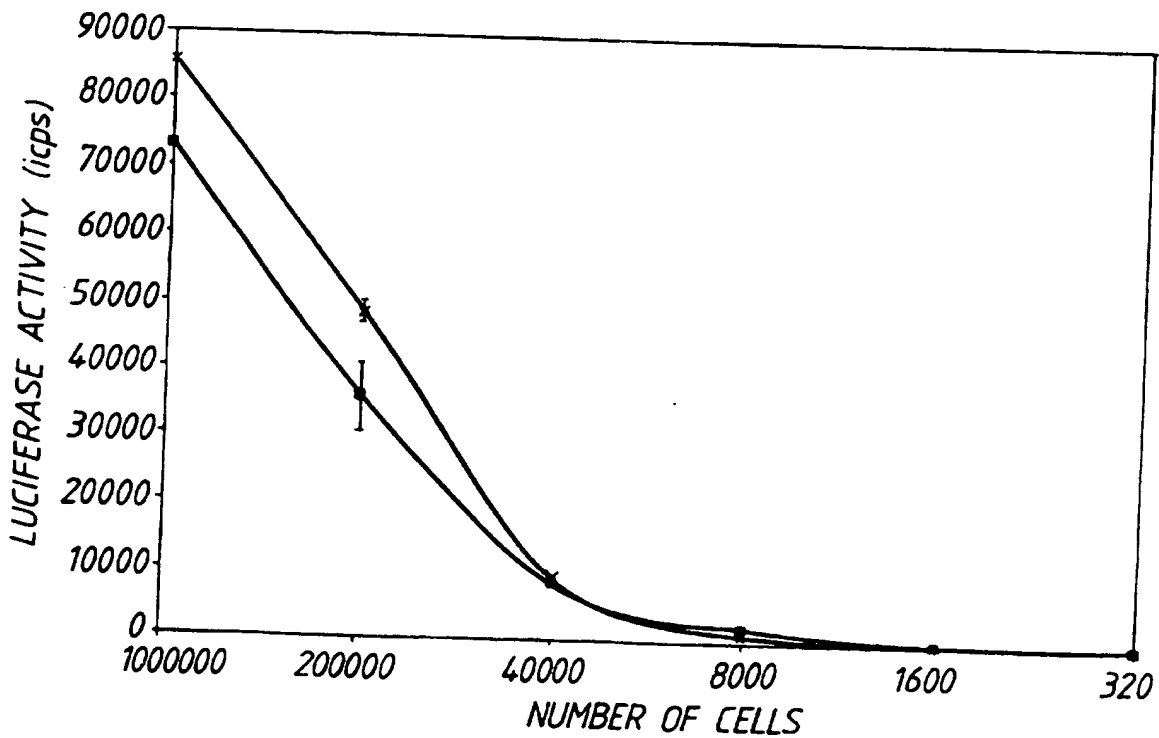


Fig.6

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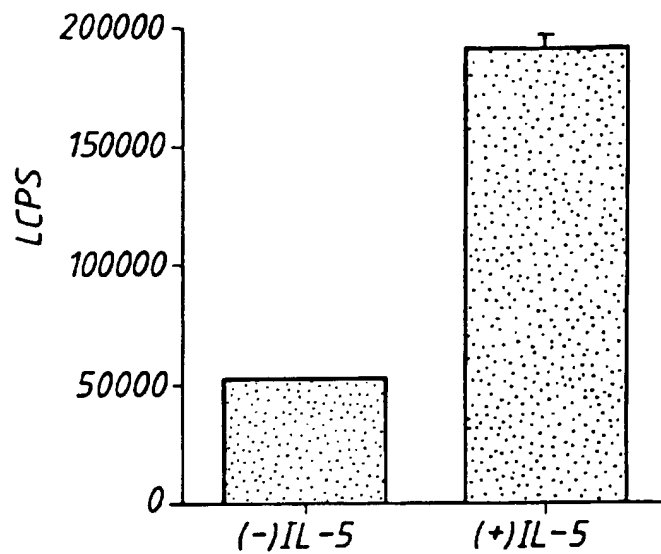


Fig.7

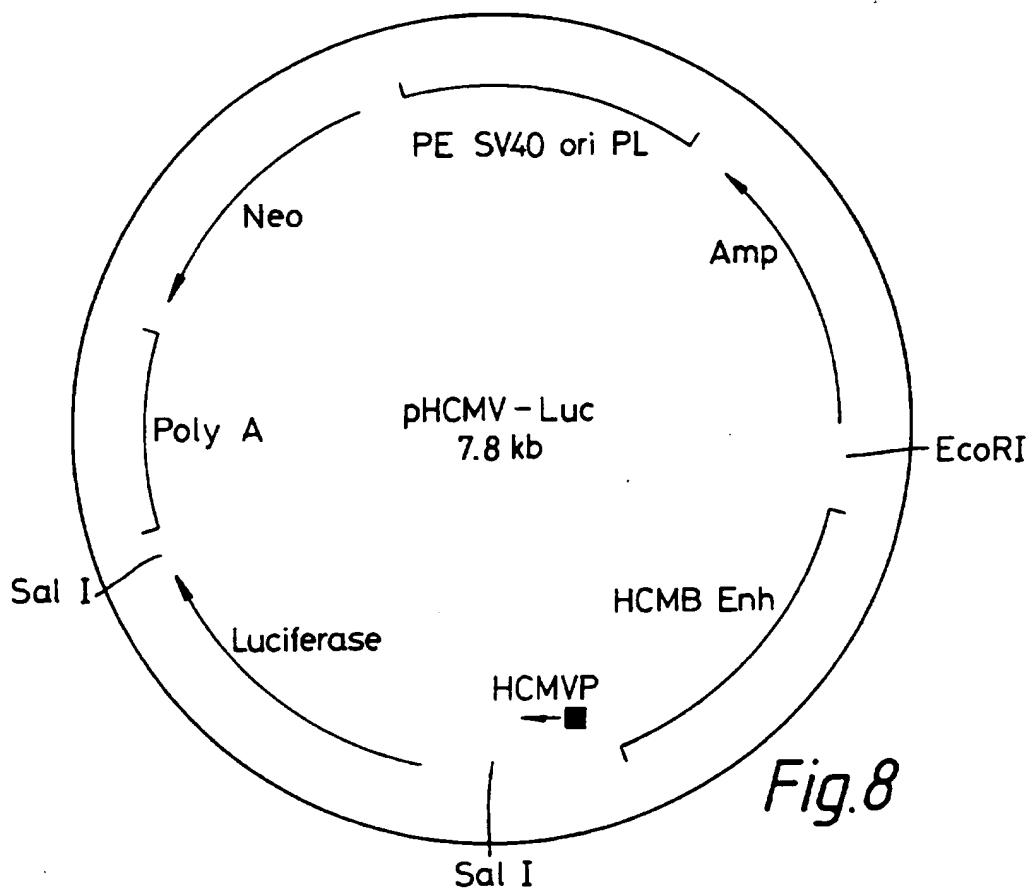


Fig.8

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 95/00692

## A. CLASSIFICATION OF SUBJECT MATTER

Int Cl<sup>6</sup>: C12N 5/10, 15/62, 15/53; C12Q 1/66

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
WPAT AND CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
JAPIO

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CHEMICAL ABSTRACTS KEYWORDS: [LUCIFERAS? (5N) (GENE# OR DNA)] AND [VIABLE OR VIABILITY OR GROWTH OR CYTOTOXIC? OR ACTIVITY]  
WPAT KEYWORDS: (See handwritten sheet(1))

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5085982 (DOUGLAS H KEITH) published 4 February 1992 See whole document	1, 5, 6, 10, 11, 22 and 23
X	DE 3833628 (GENLUX FORSCHUNGSGESELLSCHAFT FÜR BIOLOGISCHE VERFABREN mbH) published 12 April 1990 See whole document	1-26
X	Gene (1990), Volume 88, pages 197-205 (O SCHWARTZ et al.) "A microtransfection method using the luciferase-encoding reporter gene for the assay of human immunodeficiency virus LTR promoter activity" See whole Article	1, 5, 6, 10, 11, 15, 21, 22, 23, 25 and 26

☐ Further documents are listed in the continuation of Box C

☒ See patent family annex

### \* Special categories of cited documents:

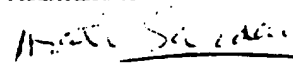
"A" document defining the general state of the art which is not considered to be of particular relevance  
"E" earlier document but published on or after the international filing date  
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)  
"O" document referring to an oral disclosure, use, exhibition or other means  
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone  
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art  
"&" document member of the same patent family

Date of the actual completion of the international search  
2 February 1996

Date of mailing of the international search report  
06 February 1996 (06.02.96)

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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 95/00692

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Biochemical and Biophysical Research Communications (1994) Volume 198, No. 2, pages 626-631 (M J CASTANON and W SPEVAK) "Functional coupling of Human Adenosine receptors to a Ligand-dependent reporter gene system" See whole Article	15, 19 and 21
X	The Journal of Steroid Biochemistry and Molecular Biology (1993) Volume 46, No. 3, pages 355-364 (E DEMIRPENCE et al.) "MVLN cells: A bioluminescent MCF-7-Derived cell line to study the modulation of Estrogenic activity" See whole Article	1, 5, 6, 10, 11, 15, 19, 21-23
X	Journal of Clinical Microbiology. (September 1993), Volume 31, No. 9, pages 2251-2254 (P W ANDREW and I S ROBERTS) "Construction of a Bioluminescent Mycobacterium and its use for Assay of Antimycobacterial Agents" See whole Article	1-26
X	Soil Biol Biochem (1991) Volume 23, No. 11, pages 1021-1024 (F A GRANT et al.) "Luminescence-Based Viable Cell enumeration of Erwinia Carotovora in the Soil" See whole Article	1, 5, 6, 10, 11, 15 and 21
X	Rapid Methods and Automation in Microbiology and Immunology (1994) pages 421-8 (P W ANDREW et al.) "Bioluminescence as a tool in mycobacteridogy" See whole article	1, 5, 6, 10, 11, 15, 22 and 23

### Information on patent family members

**PCT/AU 95/00692**

Patent Document Cited in Search Report		Patent Family Member			
US	5085982	US	873504		
DE	3833628	AU	43352/89	DE	3833628
		WO	8900626	WO	9004037

## Box

## WPAT KEYWORDS: (continued)

- (a) LUCIFERASE # AND C12N - 015/1C
- (b) (C12Q - 001/66/1C) AND (ACTIV: OR VIAB: OR GROWTH OR CYTOTOXIC: OR ADHERE:)) OR  
[(ACTIV: OR VIAB: OR GROWTH OR CYTOTOXIC: OR ADHERE:) AND (LUCIFERASE # AND  
EXPRESS:)]
- (c) [{GENE # OR DNA OR RNA) (ION) (REPORTER# OR GALACTOSIDASE)} AND EXPRESS:] AND  
[ACTIV: OR VIAB: OR GROWTH OR CYTOTOXIC: OR ADHERE:]